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A new technique for studying the stylet tracks of homopteran insects in hand-sectioned plant tissue using light or epifluorescence microscopy

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Abstract

Homopteran insects, such as aphids, psyllids and scales, inject a proteinaceous salivary sheath into their host plant tissue during feeding. This sheath, also referred to as a stylet track, remains in the tissue after the stylets are withdrawn, and is useful for studying plant resistance to insects and plant virus transmission. We describe a new method for studying stylet tracks. Hand microtome sectioned plant material was fixed and cleared in ethanol. The stylet tracks were stained with acid fuchsin and counterstained with aniline blue or fast green. The acid fuchsin stained stylet tracks were pink to red under light microscopy, and orange under TRITC epifluorescence. Stylet tracks in unstained sections autofluoresced under DAPI epifluorescence. This new technique is significantly faster and less complex than previous techniques, and permitted visualization of stylet tracks with light or epifluorescence microscopy within 1 hr of collecting fresh plant material. The technique was also applicable to a broad range of homopterans and plant taxa and provided excellent photomicrographs.

Key words: acid fuchsin, Homoptera, insect, plant material, salivary sheath, stylet track

Homopteran insects such as psyllids, aphids and whitefly feed on internal plant liquids using stylet mouthparts (Gillot 1995). In contrast to the more conspicuous effects of feeding by herbivorous insects that ingest plant structural components, the effects of homopteran feeding are more subtle. Homopterans are serious agricultural pests, however, and can significantly impact their plant hosts by (1) transmitting viruses and pathogens (de Graca 1991, Nault 1997), (2) producing honeydew that supports fungal growth on leaf surfaces (Wood et al. 1988), (3) deforming plant tissues (Williams 1994), (4) competing with plant sinks for photosynthates (Larson and Whitham 1997), and (5) removing nutrients that could be used for plant growth and reproduction (Weigert 1964).

When a homopteran insect inserts its stylets into the plant tissue, it secretes a proteinaceous salivary sheath that remains intact within the plant after the stylets are withdrawn (Miles 1968). Salivary sheaths, also known as stylet tracks, have interested scientists for over a century (Prillieux 1878, cited in Miles 1968) because they provide information on feeding mechanisms, plant resistance and virus transmission. Studies of stylet tracks usually use complex and time-consuming histological techniques that involve fixation, dehydration, paraffin embedding, sectioning, staining, and counterstaining of the plant tissue (Cohen et al. 1996, Mesfin et al. 1995, Pollard 1973, Woodburn and Lewis 1973, Davidson 1923). A technique has also been described for studying stylet tracks in unsectioned

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plant tissue (Backus et al. 1988). We introduce here a new, rapid, and simple technique to study stylet tracks in hand-microtome sectioned tissue from a broad range of plants species using light and epifluorescence microscopy.

Materials and methods

Preparation of plant tissue

Leaves or stems of various plant species including monocotyledons (*Arundo donax* L., *Monstera* sp., *Yucca* sp.), dicotyledons (*Eucalyptus globulus* Labill., *Foeniculum vulgare* Miller, *Hedera helix* L., *Nerium oleander* L.) and a gymnosperm (*Pinus* sp.) that were densely colonized by several species of homopterans including Aphididae (*Aphis fabae* complex Scop., *A. nerii* B. de F., *Cavariella aedopodii* Scop., *Cinara* sp.), Cercopidae (*Philaenus spumarius* L.), Coccoidae (*Parasaissetia nigra* Nietner), Pseudococcidae (*Pseudococcus longispinus* Targioni-Tozetti) and Psyllidae (*Ctenarytaina spatulata* Taylor, *Glycaspis brimblecombei* Moore) were collected from plants in the field and placed in plastic bags. Highly mobile species (psyllids and the cercopid) were confined in small clip cages on leaves for 1–3 weeks prior to collection to increase the density of stylet tracks in the plant tissue. After collection, the plant material was washed gently under running water to remove insects and surface debris, and was refrigerated in sealed plastic bags for up to 2 weeks prior to microtomy.

Hand microtomy

Leaf or stem pieces were sandwiched snugly in a longitudinal slit in rectangular slabs of fresh carrot that were trimmed to fit into a hand microtome. Each leaf or stem piece was oriented in the microtome so that the main veins were perpendicular to the direction of sectioning. The carrot slab supports the leaf or stem during sectioning. The plant material was cut into sections approximately 15–35 μm thick using a straight edge razor with disposable blades. As the sections were cut, they were floated off the razor blade into water in a petri dish.

Tissue fixation and clearing

Within 5–45 min after sectioning, the leaf or stem sections in the petri dish were separated from the carrot using a fine tipped paint brush or insect pin attached to the tip of a bamboo skewer, and transferred to glass vials containing 70% ethanol for fixation and clearing. The clearing time depended on the amount of chlorophyll and other pigments present in the sectioned material and how often the ethanol was replaced. To accelerate clearing, the ethanol was changed every 5–15 min. Usually, sections were adequately cleared (as indicated by the clarity of the ethanol) after 1–3 changes of ethanol. After clearing, the sections were stored in 70% ethanol.

Staining stylet tracks in cleared plant tissue (light microscopy)

Cleared sections were transferred to glass vials with known volumes (in drops) of 70% ethanol. For each 10 drops of 70% ethanol, one drop of 0.2% acid fuchsin (C.I. 62685, Lot # 914899; Fisher Scientific, Fairlawn, NJ) in 95% ethanol and glacial acetic acid (1:1, v/v) was added. The vials were shaken gently by hand for a few seconds to disperse the stain. The sections were soaked in the stain for 10–30 min before they were transferred to water in a petri dish for a few seconds and then to microscope slides for examination at ×40–1000. A paint brush, and/or bamboo skewer with an insect pin attached to one end and bent into a hook at the tip, was useful for transferring the sections from the vial. To increase the detail and contrast of the plant tissue surrounding the stylet track, sections were counterstained with 1% aniline blue (C.I. 42755, Polysciences, Warrington, PA, USA) in 95% ethanol, or 0.05% fast green (C.I. 42053, Aldrich, Milwaukee, WI, USA) in 95% ethanol. A drop of the counterstain was placed directly on a section on the microscope slide and the excess stain was washed away with several drops of 95% ethanol after 3–10 sec to obtain the desired counterstain intensity. For permanent mounts, stained sections were transferred to clean microscope slides, dehydrated with a few drops of 100% ethanol for a few seconds, then mounted in Euparal (Asco Laboratories, Gorton, Manchester, UK). Stained sections were also stored in a solution of 0.2% acid fuchsin in 70% ethanol (1:10, v/v) for several months. To prevent overstaining of the highly pigmented mesophyll in leaf sections of some species (e.g., adult leaves of *E. globulus*), the staining time or ratio of acid fuchsin stain to 70% ethanol was reduced as needed. Excess stain was also removed easily by soaking acid fuchsin stained sections in 70% ethanol as needed.

Epifluorescence microscopy

Stylet tracks stained with acid fuchsin were examined with the Nikon TRITC filter set (Ex 546/10 nm; DM 575, BA 590). In addition, cleared

unstained sections were examined for stylet track autofluorescence with the Nikon DAPI filter set (360/40 nm; DM 400, BA 460/50). Color photomicrographs were produced from both temporary and permanently mounted sections.

Results

The acid fuchsin stained stylet tracks of aphids, psyllids, scales, mealybugs, whiteflies and leafhoppers were clearly visible in a variety of host plants species of moncotyledons, dicotyledons, and gymnosperms. We observed stylet tracks within 1 hr of collecting fresh plant material. Typical examples of intact stylet tracks from the plant epidermis to the vascular tissue are shown by light microscopy in Figure 1. The fine detail of the stained stylet tracks through the epidermis (Fig. 2) and within vascular and nonvascular tissues (Fig. 3) are clearly visible. The acid fuchsin stained stylet tracks were pink to red under light microscopy, and orange under TRITC epifluorescence microscopy. Although acid fuchsin also stained various plant cells, especially the photosynthetic tissue of the mesophyll and phloem tissue, the stylet tracks were easily distinguishable due to their more intense color, characteristic shape and size, and position within the plant tissue.

Counterstaining with aniline blue or fast green increased the detail of the plant tissue surrounding the stylet tracks, especially in the unpigmented collenchyma and parenchyma tissue of the leaf mid rib and fiber cells. Aniline blue was the best counterstain for the majority of the dicotyledonous and gymnosperm species tested, while fast green was better for the highly sclerified tissues characteristic of grass species.

Under DAPI epifluorescence, stylet tracks autofluoresced in cleared, unstained sections of several plant species (Fig. 4A); however, in general more details of the stylet tracks were visible in sections stained with acid fuchsin alone (Fig. 4B) and with a counter stain (Fig. 4C). Staining the stylet track with acid fuchsin quenched the autofluorescence of the stylet tracks so that they were visible subsequently only with TRITC epifluorescence (Fig. 4D). Acid fuchsin and aniline blue appeared stable under both DAPI and TRITC epifluorescence; however fast green faded slightly within a few minutes of TRITC epifluorescence.

Discussion

Arylmethane dyes such as acid fuchsin bind to the basic amino acids of proteins (Nielson et al. 1997), and in plant histology are used to stain cellulose walls, and cortex and pith (Conn 1969). Acid fuchsin has also been used to stain various organisms in unsectioned plant tissue including fungi (McBryde 1936), nematodes (Hooper 1986), thrips eggs (Teulon and Cameron 1995) and homopteran stylet tracks and eggs (Backus et al. 1988, Ni and Wuisenberry 1997).

Our technique for studying homopteran stylet tracks in a variety of plant taxa is considerably faster and simpler than previously described techniques. In addition to the homopteran insects mentioned in this paper, this technique was used extensively to study psyllid resistance mechanisms in *Eucalyptus* (Brennan 2000). Common techniques for studying stylet tracks with light microscopy (Davidson 1923, Mesfin et al. 1995, Pollard 1973) are complex and time-consuming (Cook and Davies 1994). Paraffin embedding and thin sectioning may permit greater detail of plant cellular contents; however, based on our experience with stylet tracks of several hompteran species, sections thinner than 15–20 μm seldom contain intact stylet tracks. Cohen et al. (1996) suggested that previous methods for studying stylet track in sectioned material may chemically and mechanically disrupt stylet tracks, and they pointed out the advantages of the technique of Backus et al. (1988) that uses unsectioned plant material. Because our method involves fewer steps and uses thicker sections than the paraffin embedding techniques, there is less possibility of damaging stylet tracks.

Epifluorescence microscopy of auramine O and aniline blue stained sections has been used to visualize stylet tracks or stylet probes of aphids (Cook and Davies 1994, Traynier and Hines 1987). To our knowledge, this study is the first report of stylet track autofluorescence. Despite such autofluorescence, however, stylet tracks were most easily located and studied in acid fuchsin stained sections under light microscopy. Epifluorescence microscopy was most useful for studying acid fuchsin stained stylet tracks that were in more than one plane of focus, and to clarify the position of autofluorescent tissues such as sclerenchyma with a low affinity for acid fuchsin or the counterstains. We suggest caution in using the epiflourescence techniques with tissue stained with fast green because sections may fade after a few minutes of exposure.

Our technique for studying stylet tracks offers the following advantages over previously described techniques: it is simple, fast, applicable to both light and epifluorescence microscopy, applicable to a broad range of hompterans and plant taxa, and permits visualization of fully intact stylet tracks.

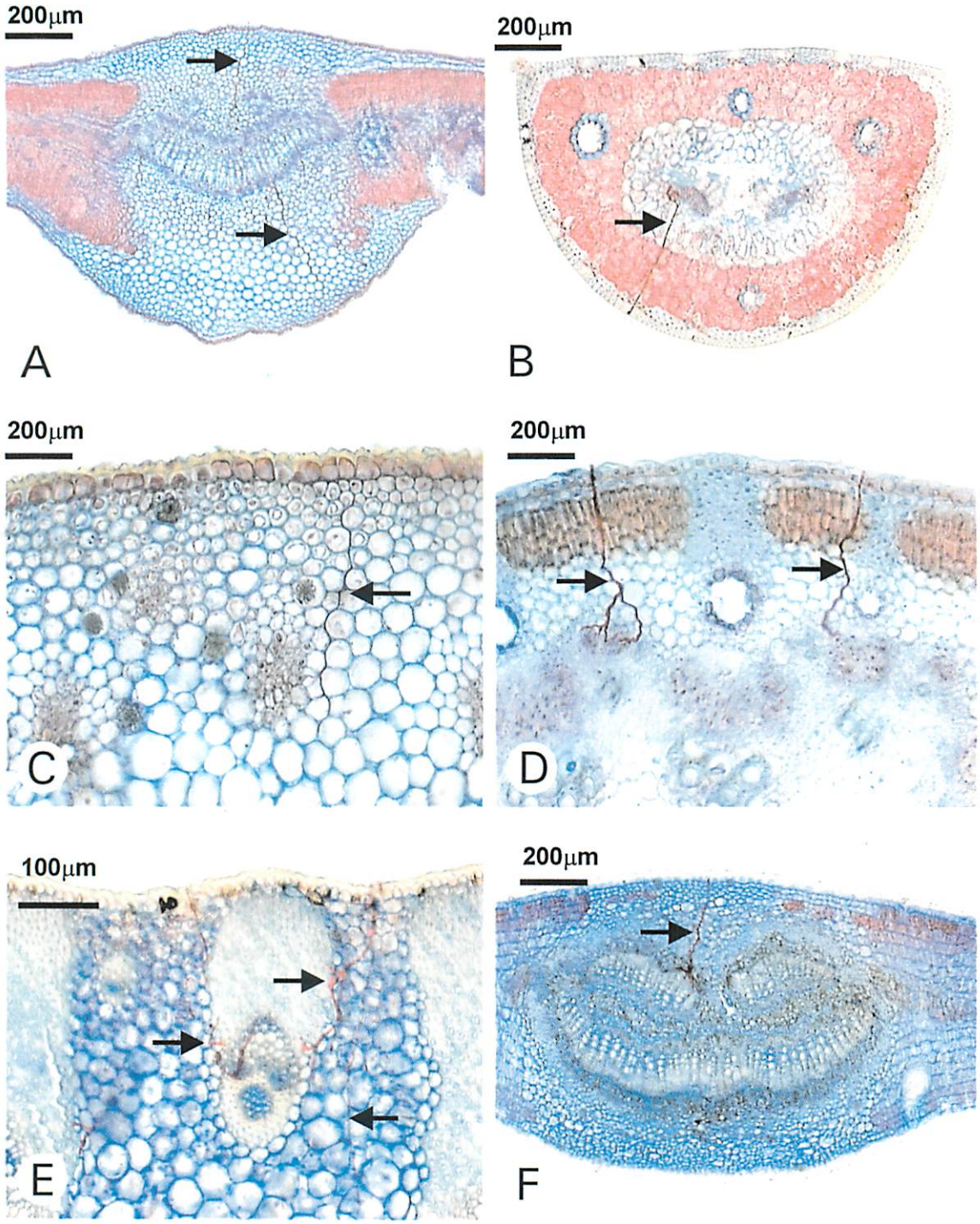

Fig. 1. Leaf and stem tissue with stylet tracks from the epidermis to the vascular tissue. Hand cut transverse sections stained with acid fuchsin and aniline blue. Light microscopy. A) Dicotyledon (*Nerium oleander)* leaf at the mid rib with intercellular stylet tracks of the aphid (*Aphis nerii*) from the adaxial (upper) and abaxial (lower) leaf surfaces. B) Gymnosperm (*Pinus* sp.) leaf with a stylet track of the aphid (*Cinara* sp.) entering the leaf through an abaxial stoma and branching into the phloem. C) Monocotyledon (*Monstera* sp.) leaf at the mid rib with an intercellular stylet track of the aphid (*Aphis fabae* complex) ending in a vascular bundle. D) Dicotyledon (*Foeniculum vulgare*) stem with two intercellular stylet tracks of the aphid (*Cavariella aedopodii*) ending in the phloem. E) Monocotyledon (*Yucca* sp.) leaf with stylet tracks of the mealybug (*Pseudococcus longispinus*) from the epidermis, along and around the sclerenchyma tissue, and ending in the vascular tissue. F) Dicotyledon (*Eucalyptus globulus*) adult leaf mid rib with a stylet track of the psyllid (*Ctenarytaina spatulata*) ending in the phloem.

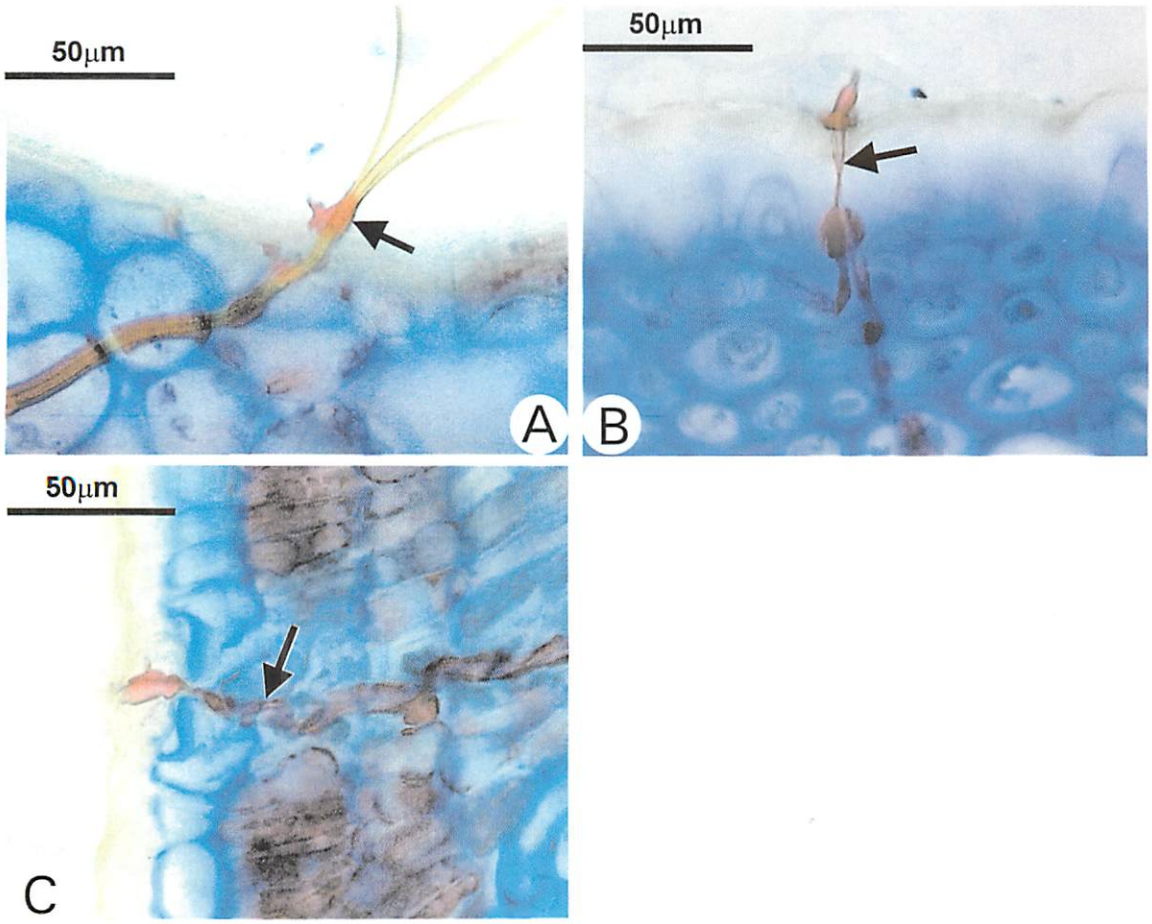

Fig. 2. Fine details of stylet tracks at the leaf epidermis. Hand cut transverse sections stained with acid fuchsin and aniline blue. Light microscopy. A) Dicotyledon (*Hedera helix*) adult leaf mid rib with the detached stylets of an individual scale (*Parasaissetia nigra*) protruding above the epidermis and within stylet track. Note the intracellular pathway of the stylet track. Above the epidermis, the mandibles and maxillae of the stylet bundle are separated because they were detached from the insect. B) *E. globulus* adult leaf at the midrib with a stylet track flange of the psyllid (*C. spatulata*) embedded in the cuticle and entering the leaf intercellularly. C) *E. globulus* adult leaf with a stylet track of the psyllid (*Glycaspis brimblecombei*) entering the mesophyll between the guard cells of a stoma.

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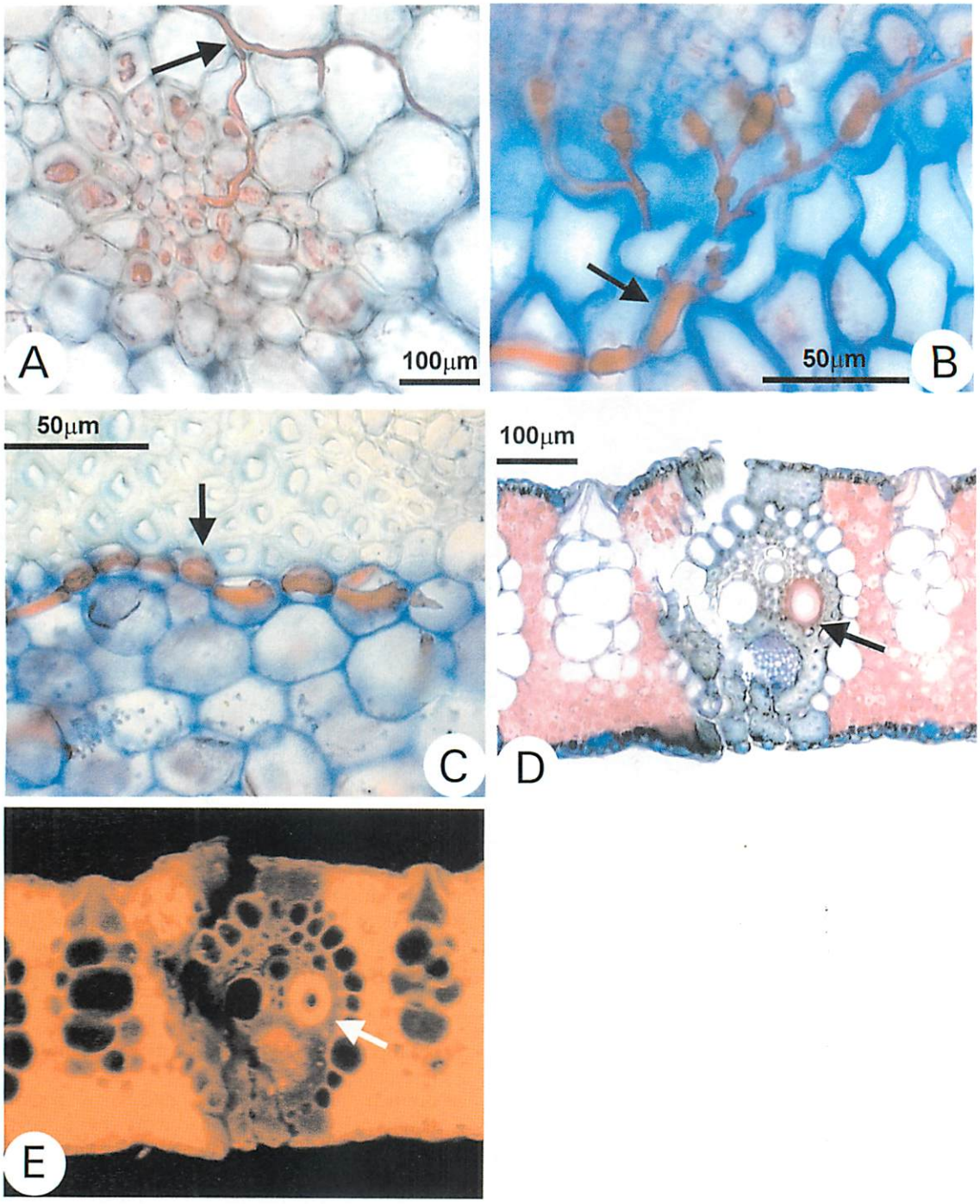

Fig. 3. Fine details of stylet tracks within vascular and nonvascular leaf tissue. Hand cut transverse sections. A) *Monstera* sp. leaf with an intercellular stylet track of the aphid (*A. fabae* complex) ending in the phloem. Acid fuchsin, aniline blue, light microscopy. B) *Pinus* sp. leaf with a highly branched stylet track of an aphid (*Cinara* sp.) in the transfusion tissue and ending in the phloem. Acid fuchsin, analine blue, light microscopy. C) *Yucca* sp. leaf with a stylet track of the mealybug (*P. longispinus*) penetrating the parenchyma cells along the vascular sclerenchyma. Acid fuchsin, analine blue, light microscopy. D) Monocotyledon (*Arundo donax*) leaf with a stylet track of the cercopid (*Philaenus spumarius*) filling the right xylem vessel of the vascular bundle. Note that the pathway from the epidermis to the xylem vessel is not apparent in this section. Acid fuchsin, fast green, light microscopy. E) Same section as (D) with TRITC filter. Acid fuchsin, fast green, epifluorescence microscopy.

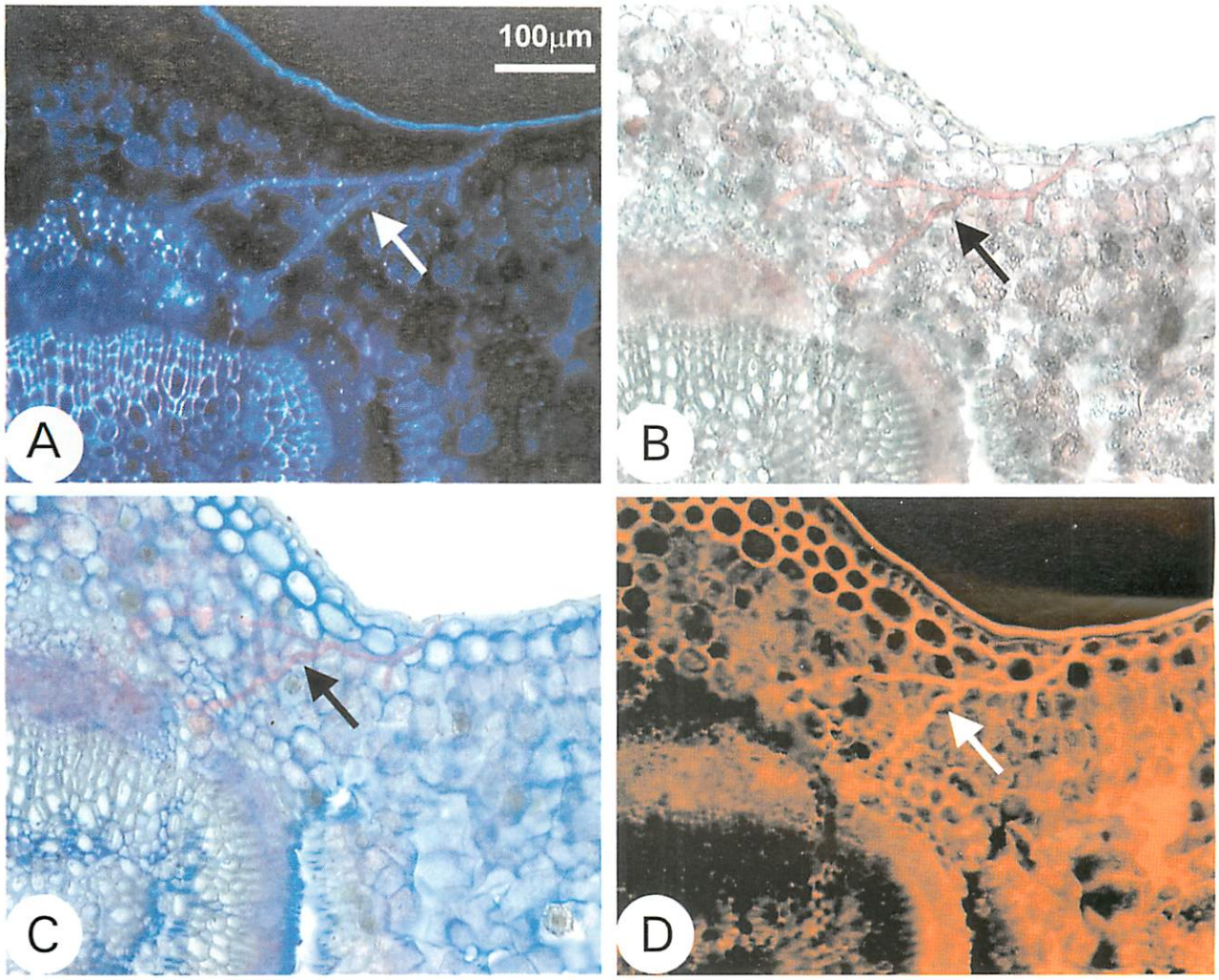

Fig. 4. A single hand cut transverse section of a *H. helix* leaf at the mid rib with a stylet track of the scale (*P. nigra*) from the adaxial epidermis to the vascular tissue with different stains under light and epifluorescence microscopy. A) No stain; only alcohol cleared. Epifluorescence microscopy with DAPI filter. B) Acid fuchsin, light microscopy. C) Acid fuchsin, aniline blue, light microscopy. D) Acid fuchsin, epifluorescence microscopy with TRITC filter.

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